THE GRAM-STAINING MATERIAL OF HUMAN EPIDERMIS*

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Twelve years after its introduction in 1884, the Gram stain was applied by Ernst for the staining of human epidermis, hair, nails and horny organs of other species (1). In his classical paper he gave a detailed account of the outstanding features of human skin stained with this method. Since that time, a number of authors applied this technic for the staining of epidermal structures and mucous membranes (2 to 8). In spite of the extensive literature, the mechanism of the stain and the nature of the Gram-positive material remain mysterious (9, 10). The study of this problem is of considerable importance for two reasons: First, there are but relatively few stains such as the Gram, which differentiate between tissue elements in the epidermis; then, in addition to bacteria and yeasts, there are only about half a dozen substances in nature which stain according to Gram (9).

In the present paper we discuss the histologic application of the Gram stain to cutaneous structures and present some new findings concerning the chemical nature of the Gram-positive material together with its possible role in epidermal differentiation.

EXPERIMENTAL

1. Histologic Studies

Deparaffinized sections were stained with the Gram-Weigert technic, as described by Johnson (8). Adequate staining could also be obtained by introducing two modifications: 1. Instead of Weigert's iodine, the use of Gram's iodine for 1 minute; 2. Decolorization with a mixture of equal volumes of acetone and 70% alcohol, followed by dehydration in graded alcohols.

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2. Measurement of Dye Retention in Pulverized Horny Material

In order to obtain a more objective measure of the extent to which various horny structures retained the dye, a semiquantitative method was developed. Quantitation of the stain has been previously attempted for bacteria (11). Instead of staining histologic sections and estimating the intensity and extent of the reaction, with this modified Gram technique we stained accurately weighed quantities of pulverized horny material and measured the dye retention colorimetrically. The steps of this method are as follows:

1. Horny material (callus, pathologic scales, hair, nails, wool, etc.) was defatted with ether and ground to standard size (60 to 80 mesh) in the Wiley mill. Ten milligram samples of this powder were weighed into glass stoppered centrifuge tubes.
2. The powdered material was stained with Stirling's aniline crystal violet (Conn's modification) (12) in a 3–5:100,000 dilution. This dye was freshly prepared from a 0.1% stock solution. The horny material was incubated for 3 minutes at room temperature in 1 ml. of the dye with frequent stirring.
3. The excess dye was washed out with 3 changes of distilled water, by centrifuging and discarding the supernate.
4. The washed material was treated with 1 ml. of Gram-Weigert's Lugol solution for 30 seconds at room temperature, immediately followed by repeated washing with water, as described above. After the final rinsing the water was thoroughly drained off.
5. The stained samples were incubated for 24 hours with 5 ml. absolute alcohol at room temperature; frequent stirring insures complete extraction of the retained dye. Then the tubes were centrifuged and the color of the supernate was measured in the Klett colorimeter at 560 mμ.

3. Effect of Chemical Agents and Enzymes on the Gram Stain

The *in vitro* method for estimating the extent of Gram-staining in pulverized horny material can be conveniently used for studying the effects of

various agents on the dye uptake. The following substances were tested:

1. Solvents: Water, alcohol, ether, acetone, 10% Duponol.

2. "Keratolytic" agents: 0.1 N NaOH, 0.1 N sodium cyanide, 5% sodium thioglycolate in 0.25 N NaOH, 75% lithium bromide, 50% urea and saturated allantoin solutions.

Oxidizing agents and acids: Ammoniacal hydrogen peroxide (10%), bromine water, concentrated nitric acid, 50% sulfuric acid, 0.4 and 3% acetic acid.

4. Metallic salts: Mercuric chloride, mercuric acetate, thallium acetate, ferric nitrate, ferric sulfate, nickel chloride, lead acetate, chromium trinitrate and trioxide and gold chloride, in 10% concentrations.

5. Enzymes: Trypsin, papain, hyaluronidase, crude testicular extract, lysozyme and ribonuclease.

6. Sulfhydryl inhibitors: Lugol's solution, saturated arsenic trioxide and 10% sodium iodoacetate.

4. Chemical Studies

Chemical determinations were performed in hydrolyzed horny material. One hundred mg. of defatted and powdered normal and pathologic horny layers, hair, nails or wool were subjected to hydrolysis with 5 ml. of 2 or 4 N HCl for periods of 10–20 hours at 105°C. The hydrolyzates were neutralized, adjusted to constant volume, decolorized with charcoal and filtered.

In the filtrates glucosamine was determined with the Elson-Morgan method, modified by Weber (13). In some instances, prior to hydrolysis, the scales were incubated with various chemical compounds. In such cases, the incubated material was washed extensively, until the washings were free of the incubating agent, as indicated by sensitive specific qualitative tests for the individual compounds.

Glucosamine was also estimated in aqueous extracts of scales (125 mg./1 ml. water). The extracts were prepared by shaking the material with water overnight at room temperature.

Sulfhydryl was determined with Bennett's reagent (14).

RESULTS

1. Histologic Findings

Our findings agree with those of previous investigators (2 to 8). In normal epidermis with a thin horny layer the stained portion is re-

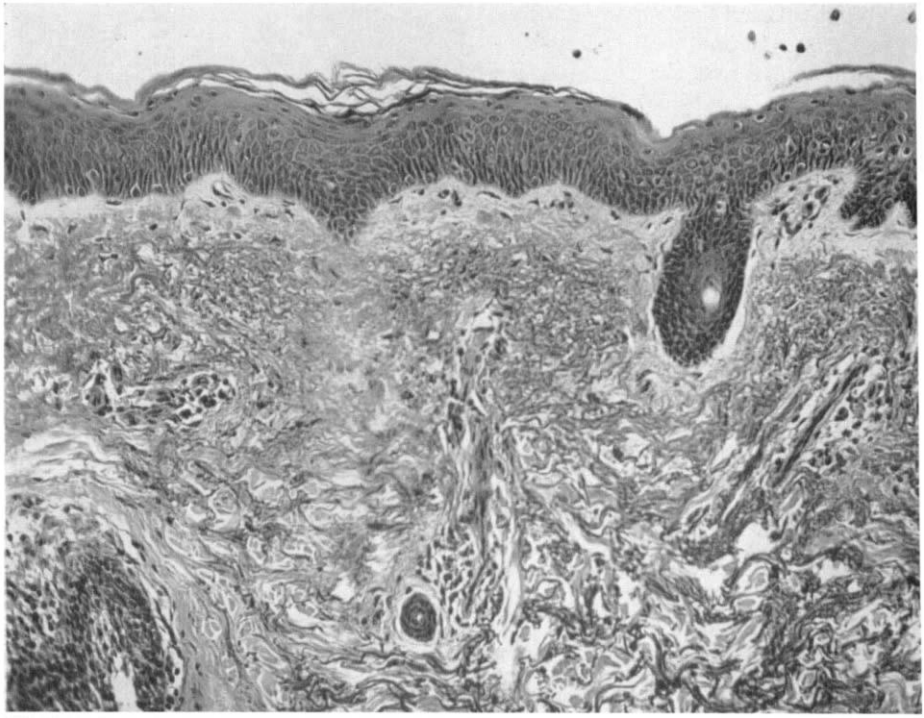

FIG. 1. Normal epidermis. Narrow Gram-positive band in transitional layers. ×165

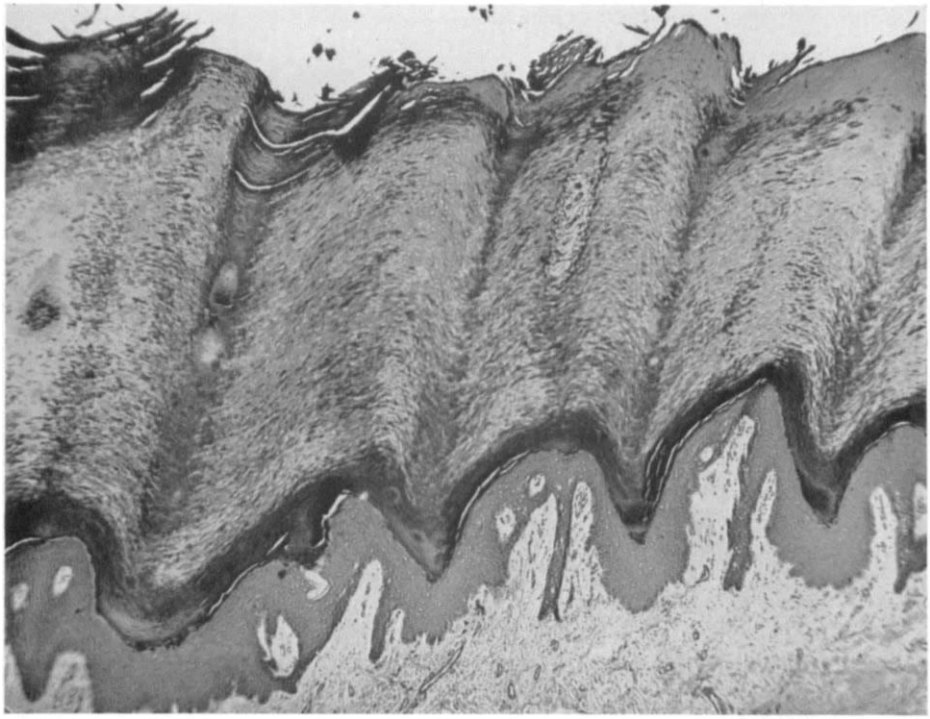

FIG. 2. Plantar epidermis. Gram-postivity extending around sweat ducts. Surface layers Gram-negative. ×40

FIG. 3. Plantar wart. Diffuse, extensive Gram-positivity. ×22

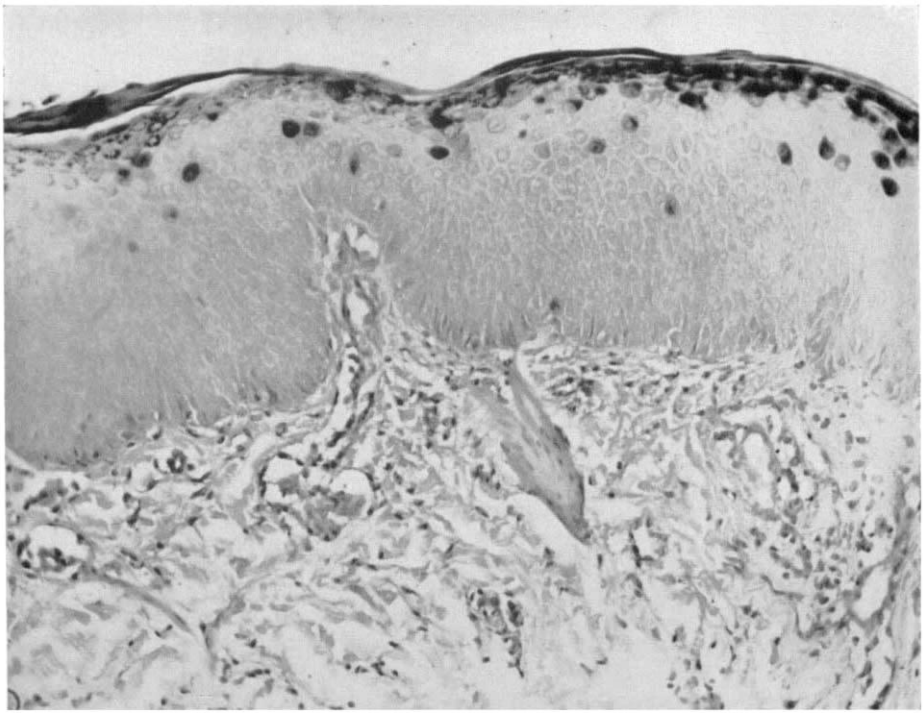

FIG. 4. Psoriatic epidermis. Scattered Gram-positive cells in upper Malpighian layers. ×105

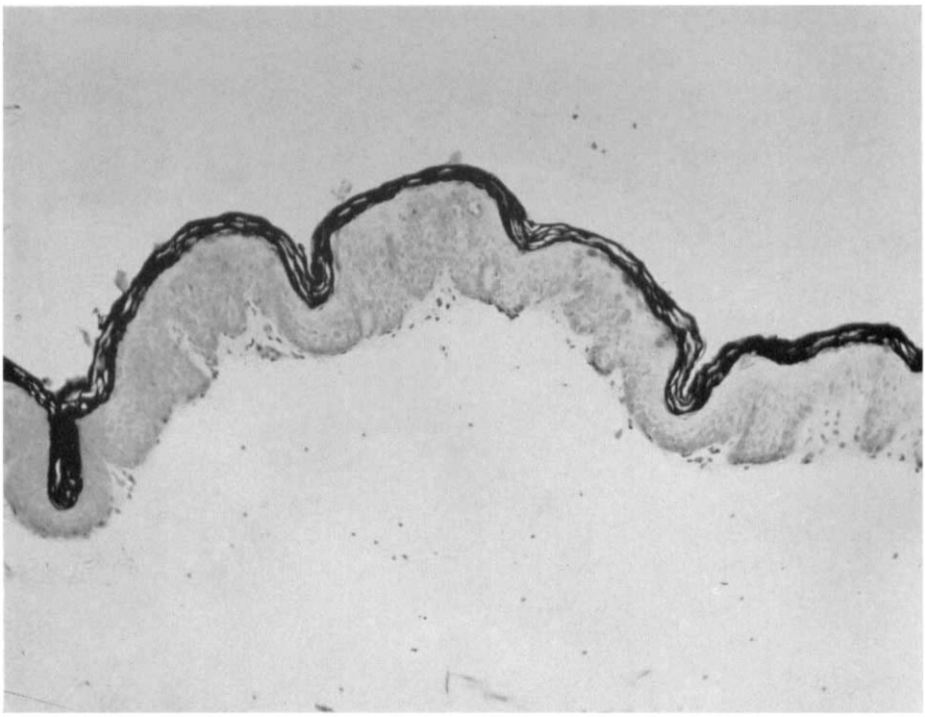

FIG. 5. Psoriatic scales. Marked Gram-staining throughout entire horny layer. ×160

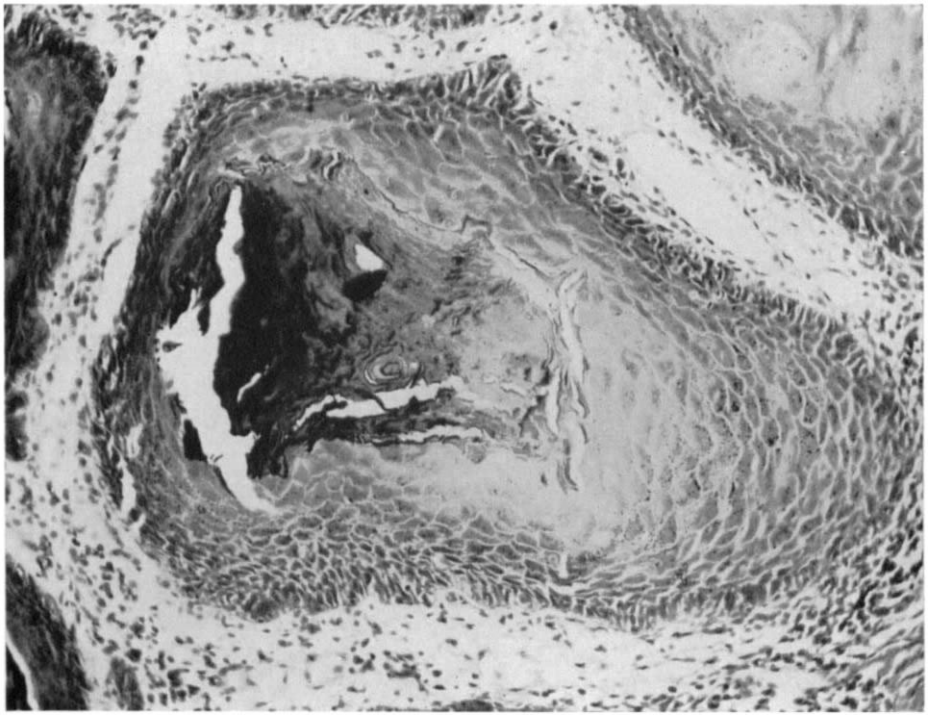

FIG. 6. Squamous cell carcinoma. Gram-positive horny pearl. ×140

stricted to a narrow band between the Malpighian and horny layers ("transitional zone") (Fig. 1.). In regions with a highly developed stratum corneum, such as the soles, the Gram stain is widely distributed. In such thick horny layers not only the lowermost zone stains, but there is scattered Gram-positivity in the more superficial layers as well, especially around the sweat ducts. However, the uppermost, most fully differentiated layers are invariably Gram-negative (Fig. 2.).

In pathologic horny layers Gram-positivity often occurs in areas with parakeratosis or dyskeratosis. Plantar warts are characterized by extensive Gram-staining (Fig. 3.). In the psoriatic epidermis some cells of the upper Malpighian layers are Gram-positive; the most intensive Gram-positivity occurs in the parakeratotic psoriatic scale (Figs. 4. and 5.). There is marked Gram-staining in the horny pearls of squamous cell carcinoma, (Fig. 6.) the dyskeratotic cells and grains of Darier's disease and the horny cells at the base of a cornu cutaneum.

2. In Vitro *Studies of the Gram-Positive Substance*

The increased affinity of certain pathologic horny layers to the Gram-stain was confirmed with our semiquantitative test. Pulverized callus takes up very little of the dye, while psoriatic scales are highly Gram-positive (Fig. 7.). A high degree of Gram-positivity was found in scales from patients with non-psoriatic exfoliative dermatitis and Darier's disease, while horny layers of patients with keratoderma plantare and ichthyosiform erythroderma gave the same low range of colorimetric readings as callus.

In order to investigate the chemical nature of the Gram-positive material, the simplest method would be to destroy this substance and to analyze the horny material before and after abolition of the Gram-stain. However, when we tried to adopt such procedures, the Gram-staining resisted most of our attempts at its destruction. The resistance of Gram stained keratohyalin against various enzymes has been commented upon by Montagna (15). To our surprise, the

staining proved resistant to almost all other chemical influences as well. It could not be abolished by any of the solvents, "keratolytic" and oxidizing agents, acids, enzymes and sulfhydryl inhibitors tested by us. The only substances which consistently blocked the staining were certain metallic salts, namely mercuric acetate and ferric salts; the effect of chromium salts appeared less pronounced. These salts, especially mercuric acetate, were of great help in

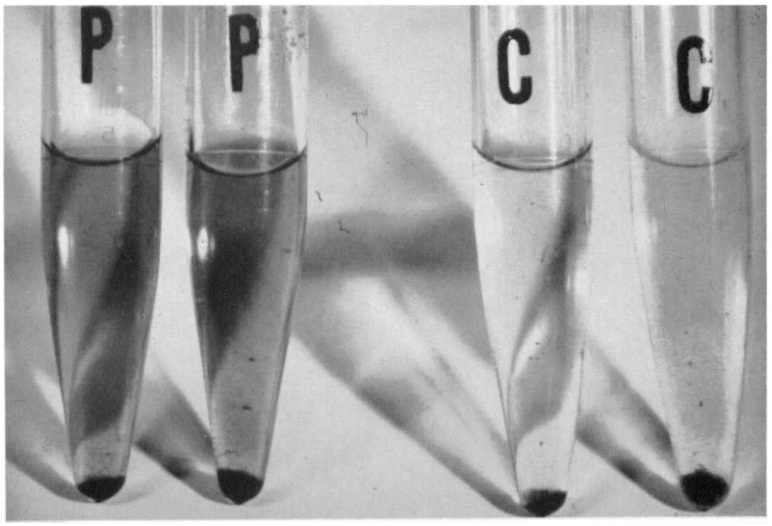

FIG. 7. In vitro Gram stain of pulverized horny layers. Marked staining of psoriatic scales (P), weak staining in callus (C).

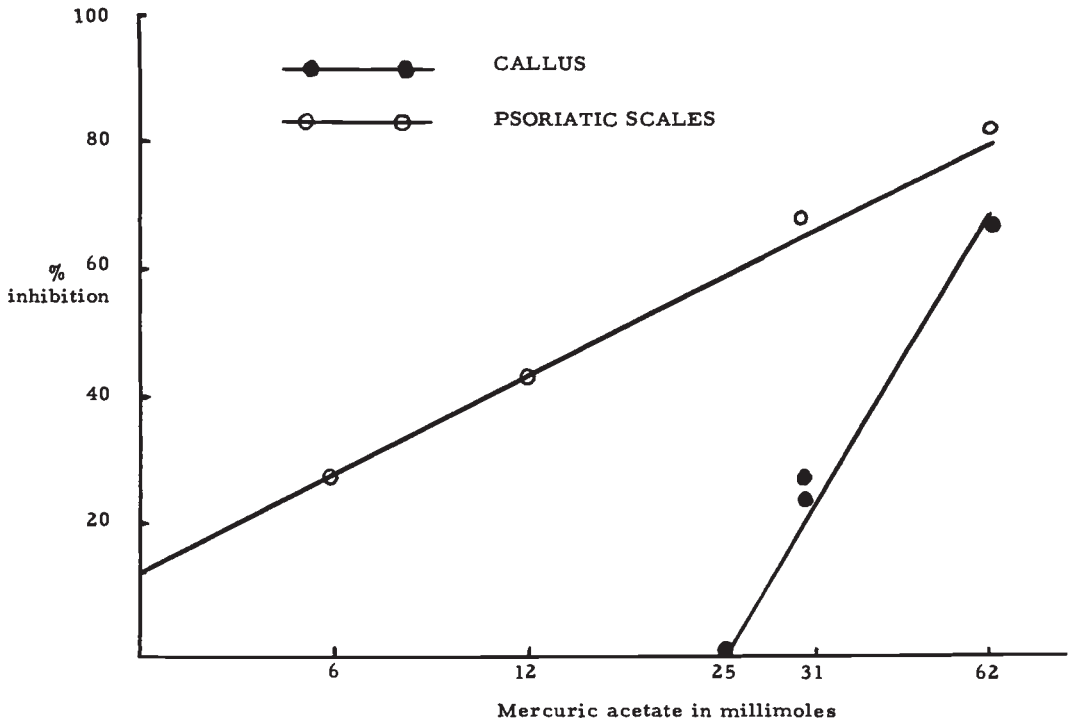

FIG. 8. Inhibition of Gram stain by increasing concentrations of mercuric acetate.

advancing our understanding of the possible nature of the Gram-positive substance.

3. Effect of Mercuric Acetate on the Gram-Stain

When pulverized horny material is incubated with 10% mercuric acetate, its affinity to the Gram-stain becomes completely lost. Mercuric chloride has a similar, but less pronounced effect. The blocking affects the first step in the Gram-reaction, the uptake of crystal violet; inhibition of this step was strikingly apparent with our semiquantitative method. Therefore the blocking cannot be attributed to the formation of the insoluble mercuric iodide in the second step of the reaction. The inhibition can be reproduced in histologic sections as well. However, mercuric ions do not influence the metachromasia of such sections. In a 10% solution, mercuric acetate interferes also with the staining of heat-killed Gram-positive pneumococci.

With lower concentrations the inhibition is incomplete. When the per cent inhibition is plotted against varying concentrations of mercuric acetate on semilog paper, a straight-line correlation can be obtained (Fig. 8.).

4. Glucosamine Content of Horny Structures; Effect of Mercuric Acetate

All horny structures studied by us yielded significant amounts of glucosamine upon hydrolysis. The Elson-Morgan method for the determination of this substance is quite specific. The only possible source of error may be the false positive reaction given by mixtures of amino acids and reducing sugars in hydrolyzed tissues (16). However, these components may be distinguished from glucosamine by hydrolysis of the tissue with 4 N HCl for 16 hours which eliminates them completely, while leaving most of the glucosamine intact. In such a way we were able to ascertain that the material in our hydrolyzates was glucosamine, a building stone of mucopolysaccharides.

The glucosamine contents of hydrolyzed horny structures are listed in Table I. It appears that there is a parallelism between the degree of Gram-positivity and the glucosamine concentration. The definite establishment of a correlation is at present precluded by the inaccuracies inherent in the semiquantitative Gram-method.

The amounts of extractable free and bound glucosamine have been published in a previous paper (17). The exact quantities are difficult to assess; the hydrolysis test for the elimination of the false positive components cannot be used, because it releases further large amounts of glucosamine. Nevertheless, it seems that both the bound and free extractable fractions are increased in some pathologic scales, such as psoriasis or exfoliative dermatitis. With the exception of our findings in callus, our data also generally agree with those of Weber and Braun-Falco (18).

Trypsin and crude testicular extract had no effect on the amounts of glucosamine released by hydrolysis. Among the "keratolytic" agents, sodium sulfide and cyanide had no effect, while sodium hydroxide and sodium thioglycolate occasionally reduced the quantities of glucosamine recovered after hydrolysis.

However, the effect of mercuric acetate upon the glucosamine content was absolutely consist-

TABLE I

Glucosamine in Hydrolyzates of Horny Structures

Horny tissue	Glucosamine in mg/100 Gm	Gram stain
Callus (extracted with water)	#1 120 #2 83 #3 100 #4 60 #5 60 #6 60 #7 110 #8 110 #9 75	Very weak
Ichthyosiform erythroderma (extracted with water)	130, 170, 180	Very weak
Exfoliative dermatitis (extracted with water)	320, 250, 280	Moderate
Psoriasis (extracted with water)	#1 260 #2 270, 290, 200 #3 280, 290 #4 120 #5 480, 450 #6 580 #7 160, 130, 160	Moderate to intense
Hair	110	
Wool	120	Weak to moderate
Nails	100	

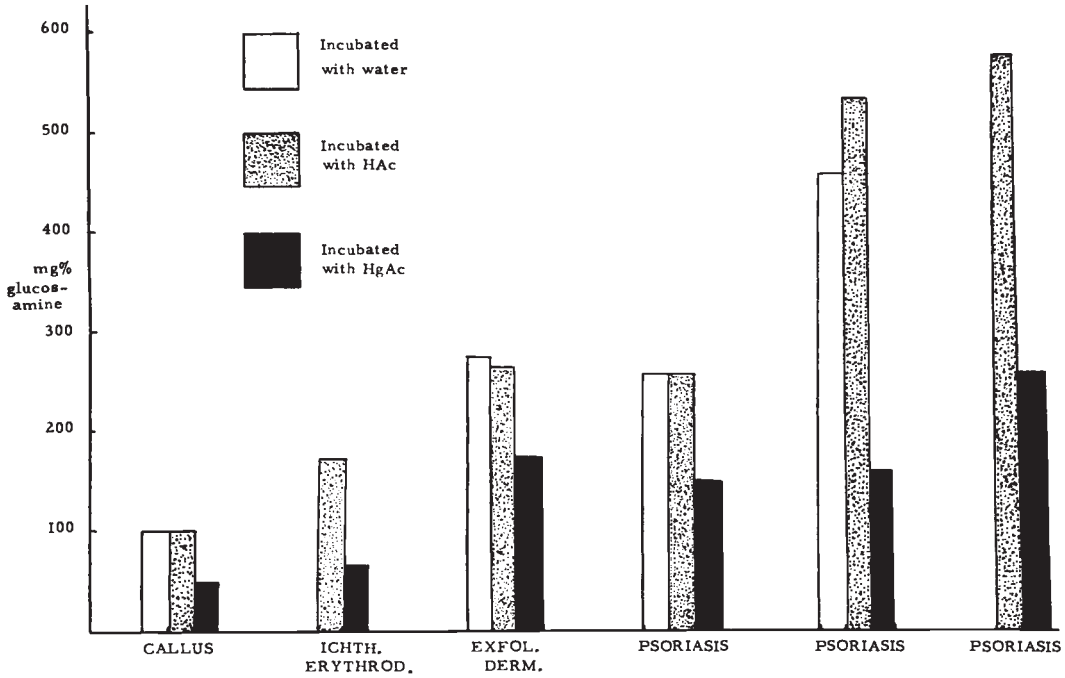

FIG. 9. Glucosamine content of hydrolyzed scales after pretreatment with water, acetic acid and mercuric acetate.

ent. Whenever, prior to hydrolysis, either normal or pathologic ground scales, pulverized hair or nails were incubated with 10% mercuric acetate, their glucosamine content was sharply reduced, as compared with controls, treated with water or an equimolar solution of acetic acid (Fig. 9.).

In the course of these glucosamine estimations it was observed that when the hydrolyzates were boiled with the alkaline acetyl-acetone reagent, a precipitate was formed. This precipitate dissolved upon the subsequent addition of the acid Ehrlich-reagent in the second step of the color reaction. No such precipitation occurred in any of the other hydrolyzates of scales which had been pretreated with keratolytic or oxidizing agents, enzymes or sulfhydryl inhibitors. This phenomenon led us to believe that the precipitate formed in the hydrolyzates of scales which had been pretreated with mercuric acetate, contained the major part of the glucosamine in a non-reactive form. This assumption was confirmed by a simple experiment. Prior to the determination of glucosamine, the hydrolyzates were boiled in an alkaline medium, in order to bring about maximum precipitation. The precipitate was subsequently separated by centrifugation and when glucosamine was estimated in the supernate, none could be found. In preliminary experiments with scales pretreated with ferric sulfate, we found a similar reduction in the glucosamine content. However, in these hydrolyzates the little precipitate formed upon alkalinization was considerably less than in the scales pretreated with mercuric acetate.

It was considered that although the scales had been extensively washed after their incubation with mercuric acetate, inorganic mercury remained in the hydrolyzates and was precipitated upon alkalinization. This inorganic precipitate could have carried down the glucosamine. Such an error was ruled out by adding to hydrolyzates of untreated scales relatively large amounts of mercuric acetate, prior to glucosamine determination. Under these conditions there was relatively slight reduction in the glucosamine content (Table II.).

We hoped to isolate the Gram-reactive substance from the hydrolyzates of scales pretreated with mercuric acetate. For this purpose we prepared precipitates on a large scale from the alkalinized hydrolyzates. However, even after extensive purification, the washed precipitates

TABLE II

Glucosamine Content of Hydrolyzed Psoriatic Scales: Effect of Various Agents Added before and after Hydrolysis

(Glucosamine in mg/100 Gm dry defatted tissue)

	Treatment of scales:				
	Before hydrolysis:			After hydrolysis:	
	Water	Acetic acid	Mercuric acetate	Acetic acid	Mercuric acetate
Case #1	480	520	164	590	450
Case #2	270	270	94	370	210
Case #3	150	140	65	190	130

were still contaminated with large amounts of inorganic mercury. Attempts to remove every trace of inorganic mercury by treating the scales, prior to hydrolysis, with chelating agents, were unsuccessful. Isolation of the material from hydrolyzates of scales pretreated with ferric sulfate proved equally disappointing. At present, other methods for the recovery of the Gram-positive substance are being tried.

DISCUSSION

The Gram-positivity of microorganisms and tissues is governed by physical and chemical factors, which are incompletely understood (8, 9, 10). Attempts to attribute the Gram-staining to single chemical components alone, proved incorrect. Thus, it has been repeatedly assumed that Gram-positivity in the epidermis was due to sulfhydryl compounds (3, 4, 5, 19). Essentially, this belief is based on three lines of evidence: 1. The similar distribution of Gram-positive and sulfhydryl reactive areas in the epidermis; 2. the alleged abolition of Gram-positivity by sulfhydryl inhibitors and 3. the conversion of Gram-negative structures to Gram-positivity by procedures which liberate or create sulfhydryl groups, such as reducing agents in hair (6) or various denaturing measures in wool (20, 21).

The first evidence is circumstantial and irrelevant. Some tissues, such as powdered nail, hair or wool, are quite poor in SH, yet are Gram positive. The alleged prevention of Gram-staining by sulfhydryl inhibitors could not be confirmed by a number of authors (6–8, 22); we also were unable to block the Gram-stain with sulfhydryl inhibitors, such as iodoacetate, iodine or arsenicals. Completeness of inactivation of sulfhydryl groups was proved by chemical assays. The inhibition of the stain by mercuric salts, as described above, is not based on sulfhydryl-inhibition, because other heavy metallic salts which do not inhibit sulfhydryl groups may have a similar effect.

In our opinion, the third evidence, the puzzling conversion of hair and wool to Gram-positivity by reducing and denaturing agents, is based on correct observations and incorrect interpretations. Thioglycolate and denaturing agents not only create or liberate sulfhydryl groups, but also increase the permeability of the tissue and promote the entrance of the dye. As mentioned before, Gram-positivity is dependent on physical as well as chemical factors. Enhanced penetration of the dye may be the crucial factor in converting Gram-negative structures to Gram-positivity.

This assumption is supported by the following simple experiment: While virgin wool takes up very little of the stain, grinding of the same wool prior to staining, greatly increases its Gram-staining ability. By the same token, in converting Gram-negative structures to Gram-positivity, the main role of thioglycolate may be to facilitate the access of the dye into the interior of the tissue where the Gram-positive compounds are located.

Elimination of the sulfhydryl compounds as contenders for the role of Gram substrates leaves this substance still unidentified. The observations described in this paper bring indirect evidence in support of the theory that the Gram-positive substance of human epidermis and epidermal structures may be a complex, highly resistant acid mucopolysaccharide. Evidence for this theory may be summarized as follows:

1. Upon hydrolysis, Gram-positive horny structures yield varying amounts of glucosamine. The quantities of glucosamine released from various horny layers roughly parallel the degree of Gram-positivity of these structures.

2. Mercuric and other heavy metallic salts which block the first phase of the Gram-staining procedure, greatly decrease the amounts of reactive glucosamine made available by hydrolysis. Precipitates formed upon alkalinization of such hydrolyzates contain the available glucosamine. The mechanism of action of these metals is obscure. Possibly they combine with the Gram-positive material in such a way that

the dye-uptake, as well as the reactivity of the glucosamine building stones is blocked.

3. Gram-positive bacteria have been shown to contain ten times more hexosamine than their Gram-negative counterparts (10, 23); as in the epidermis, mercuric salts inhibit the Gram-reaction in bacteria as well.

The observed phenomena may be explained by the following provisional working theory: Under normal conditions, the epidermal cells produce a highly resistant Gram-positive mucopolysaccharide which is precipitated in or around the keratohyalin granules. As the horny layer is formed, this material is decomposed to such an extent that even its building stones, such as glucosamine, largely disintegrate in the fully developed stratum corneum. In parakeratotic and many types of pathologic keratinization and in coherent horny structures, such as hair or nails, this decomposition is incomplete. The Gram-positive mucopolysaccharide is excreted into the horny layer; its building stones may be recovered from it either by hydrolysis or, in pathologic scales, even in a free state. The increased glucosamine content of psoriatic scales is therefore another characteristic, though non-specific chemical feature of the disease.*

The practical implications of our findings are in the experimental stage. Attempts to interfere with the abnormal mucopolysaccharide metabolism by injecting other mucopolysaccharides into the psoriatic lesions were all unsuccessful. The beneficial effect of locally injected heparin, as reported by Eberhartinger, must be discounted, because of the hemorrhagic side-effects and the enormous doses administered (30).

The assumption that such an extremely resistant, glucosamine containing material occurs in all highly Gram-positive human epidermal structures, (pathological horny layers, hair and nails) raises some interesting possibilities. It is conceivable that the "cohesion" of these structures is at least partly due to this hypothetical substance. To the functions of the epidermal cell, its multipotentiality to produce keratin or sebum, we will have to add a third ability: the manufacturing of mucopolysaccharides. The quantity, quality, or both, of the epidermal mucopolysaccharides may determine some of the physical aspects of the horny structures formed. The extreme resistance of the Gram-positive substance to all kinds of chemical and enzymatic agents leaves the field wide open for further research.

SUMMARY

1. The nature and possible role of the Gram-positive material of human epidermis and horny structures were studied with histological and chemical methods.

2. In sections of human epidermis, the lowermost horny layers are Gram-positive; in many cases of pathologic keratinization there is diffuse and extensive staining of the horny layer throughout its entire thickness.

3. For the objective study of the Gram-reaction a semiquantitative colorimetric assay was developed, by staining weighed amounts of pulverized horny structures *in vitro*. The dye taken up by the horny material was extracted with alcohol and its intensity estimated colorimetrically. Significant amounts of dye combined with pulverized hair, nails, wool and some pathologic scales, while powdered callus was relatively Gram-negative.

4. The Gram-stain could not be abolished by

* In his recent paper on the histochemical aspects of parakeratoses, Steiner (7) writes as follows: "The SH content of hyperkeratotic lesions seems greater than that of parakeratoses. . . . If this finding can be confirmed by quantitative methods, it would throw doubt on the conclusions of Flesch and Esoda, according to which abnormally increased SH values of the stratum corneum are characteristic of psoriatic parakeratosis (24). Magnus has already found high SH values not only in the scales of psoriasis, but also in seborrheic and psoriasiform dermatitis." (25). This statement implies that we attributed to the high SH content of psoriatic scales a specific role in the psoriatic epidermal process and that Magnus drew attention to the high SH occurring in other scaling diseases. This implication is at variance with out views, expressed in a number of publications, including the one quoted by Steiner (24, 26, 27, 29). Already the first author, Zingsheim, who observed the high SH content of psoriatic scales with histochemical methods, emphasized its non-specific nature (28); on the basis of our quantitative studies, we had no reason to say otherwise. Our latest statement on this topic is quite explicit: "The high sulfhydryl content is the least specific of the chemical changes studied by us. Whenever the horny layer is formed at a more rapid rate, its sulfhydryl content rises." (29). The basis of the misunderstandings may lie in semantics: While all the chemical changes described by us and others are characteristic for psoriasis, i.e. they occurred in all psoriatics studied by us, they are not uniquely so: in other words, they are not *specific* for this disease, as they were found in other scaling conditions as well.

a large variety of solvents, keratolytic and oxidizing agents, acids, enzymes or detergents. Sulfhydryl-inhibitors had no effect on the Gram-stain.

5. Certain salts of heavy metals, especially mercuric acetate and ferric sulfate in adequate concentrations, completely blocked the uptake of stain by horny material. The inhibition affected the first step of the Gram-reaction. Similar inhibition was obtained in heat-killed pneumococci.

6. Glucosamine determinations in hydrolyzed horny material revealed considerable quantities of this substance in normal and pathologic horny layers, nails, hair and wool. There seemed to be a parallelism between the Gram-reactivity of these structures and their glucosamine content.

7. Pretreatment with mercuric acetate greatly lowered the amounts of demonstrable glucosamine in the hydrolyzates of horny structures. This phenomenon was attributed to the combination of mercuric ions with the Gram-positive material in such a way that the dye-uptake, as well as the reactivity of the glucosamine building stones were blocked through the formation of non-reactive mercuric "salt." Attempts to isolate this compound in a pure form thus far were unsuccessful.

8. These findings suggest that epidermal cells are capable of synthesizing a highly resistant, glucosamine-containing material, probably a mucopolysaccharide. In normal epidermal development this material is largely decomposed, to such an extent that even its building stones, such as glucosamine, mostly disintegrate in the fully developed horny layers. In parakeratotic and many types of pathologic keratinization and in coherent horny structures, such as hair or nails, this decomposition is incomplete.

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ADDENDUM

After completion of this manuscript our attention was called to Fischer and Zaleschuk's semi-micromethod for estimating the amounts of crystal violet taken up by biological material (J. Histochem. Cytochem., **6:** 237, 1958). These authors found that nail takes up considerable amounts of crystal violet, about as much as alkali degraded wool. Quantitative data on Gram stain taken up by nails were not given.

REFERENCES

1. Ernst P.: Studien über normale Verhornung mit Hülfe der Gramschen Methode. Arch. f. mikr. Anat., **47:** 669–706, 1896.
2. Wolf, J.: Die innere Struktur der Zellen des Stratum desquamans der menschlichen Epidermis. Jahrb. f. Morphol. u. mikr. Anat. 2. Abt., **46:** 170–202, 1939.
3. Steigleder, G. K.: Zum histochemischen Nachweis SH-und S-S gruppenhaltiger Substanzen in der normalen und pathologisch veränderten Haut des Menschen. Klin. Wchnschr., **34:** 495–6, 1956.
4. Steigleder, G. K.: Die Histochemie der Epidermis und ihrer Anhangsgebilde. Arch. klin. exp. Dermatol., **206:** 276–317, 1957.
5. Steigleder, K. K.: Morphologische und histochemische Befunde in pathologischer Hornschicht, insbesondere bei Parakeratose. Ein Beitrag zur Biologie der Hautoberfläche. Arch. klin. exp. Dermatol., **207:** 209–29, 1958.
6. Siebert, M.: Beitrag zur Histochemie der normalen und pathologischen Verhornung. Dermat. Wchnschr., **137:** 673–81, 1958.
7. Steiner, K.: A histochemical study of hyperkeratoses (Nucleic acids, carbohydrates and sulfur compounds.) Arch. Dermat. & Syph., **79:** 436–43, 1959.
8. Johnson, P. L., Hoffman, H. and Rolle, G. K.: The Gram staining mechanism of cat tongue keratin. J. Histochem. Cytochem., **5:** 84–90, 1957.
9. Bartholomew, J. W. and Mittwer, T.: The Gram stain. Bact. Rev., **16:** 1–29, 1952.
10. Work, E.: Biochemistry of the bacterial cell wall. Nature, **179:** 841–7, 1957.
11. Mittwer, T.: Semiquantitative evaluation of the Gram reaction. Science, **128:** 1213, 1958.
12. Lillie, R. D.: Histopathologic Technic and Practical Histochemistry. New York and Toronto, The Blakiston Co., 1954.
13. Weber, G.: Vergleichende Untersuchungen über das quantitative Verhalten proteingebundener Kohlenhydrate in Blutserum bei Dermatosen. Acta dermat-Venereol., **38:** Suppl. 38, 1958.
14. Flesch, P. and Kun, E.: A colorimetric method for determination of sulfhydryl groups in tissue homogenates by 1-(4-chloromercuriphenylazo)-naphthol-2. Proc. Soc. Exper. Biol. & Med., **74:** 249–51, 1950.
15. Montagna, W.: Structure and Function of Skin. New York, Academic Press, 1956.
16. Kent, P. W. and Whitehouse, M. W.: Biochemistry of the Aminosugars. p. 181. London, Butterworths Scientific Publications, 1955.

17. Flesch, P.: Mucopolysaccharides in human epidermis. J. Soc. Cosm. Chem., **10:** 154–9, 1959.
18. Weber, G. and Braun-Falco, O.: Ueber das Vorkommen eines sauren Glykoproteids in Psoriasisschuppen. Dermat. Wchnschr., **138:** 789–93, 1958.
19. Fischer, R.: The selectivity of the Gram stain for keratins. Experientia, **9:** 20–1, 1953.
20. Fischer, R. and Larose, P.: Contribution on the behavior and structure of the cytoplasmic membrane of bacteria. Canad. J. Med. Sci., **30:** 86–105, 1952.
21. Larose, P. and Fischer, R.: Reversal of Gram-staining behavior. Science, **117:** 449–51, 1953.
22. Libenson, L. and McIlroy, A. P.: On the mechanism of the Gram stain. J. Infect. Dis., **97:** 22–6, 1955.
23. Bettelheim-Jevons, F. R.: Protein-carbohydrate complexes. Adv. Protein Chemistry, **13:** 35–105, 1958.
24. Flesch, P. and Esoda, E. C. J.: Deficient water-binding in pathologic horny layers. J. Invest. Dermat., **28:** 5–13, 1957.
25. Magnus, I. A.: Observations on the thiol content of abnormal stratum corneum in psoriasis and other conditions. Brit. J. Dermat., **68:** 243–51, 1956.
26. Van Scott, E. J. and Flesch, P.: Sulfhydryl groups and disulfide linkages in normal and pathological keratinization. Arch. Dermat. & Syph., **70:** 141–54, 1954.
27. Flesch, P. and Esoda, E. C. J.: Defective epidermal protein metabolism in psoriasis. Arch. Dermat. & Syph., **76:** 393–401, 1957.
28. Zingsheim, M.: Die Rolle freier Sulfhydrylgruppen in der Schuppenflechte. Deutsche med. Wchnschr., **77:** 1630–1, 1952.
29. Flesch, P. and Esoda, E. C. J.: Chemical changes in psoriatic scales. Ann. New York Acad. Sc., **73:** 989–99, 1958.
30. Eberhartinger, C.: Beitrag zur Heparintherapie der Psoriasis vulgaris. Dermat. Wchnschr., **134:** 1369–74, 1956.

DISCUSSION

Dr. Walter F. Lever (Boston, Mass.): Dr. Braun-Falco has demonstrated in histologic sections that the surface of the epidermal cells as well as the intercellular bridges are coated with an acid mucopolysaccharide (Arch. klin. exp. Derm. **207:** 459, 1958). I wonder if this acid mucopolysaccharide is in any way correlated with the material that you have found to be gram-positive.

Dr. Eugene J. Van Scott (Bethesda, Md.): When dealing with the epidermis either by direct chemical methods or histochemically, how can one identify substances brought up from the corium, substances which might circulate quite freely between the epidermal cells and which might be non-specifically absorbed onto proteins or other chemical structures of the epidermis?

Dr. John M. Knox (Houston, Texas): I would like to congratulate Dr. Flesch on this work. In recent years there has been more and more interest in chemical means of diagnosis, as well as in histological diagnosis.

Histochemistry is being widely studied. We are trying to adapt chemical changes that occur in scales and the epidermis into a diagnostic aid. Dr. Ogura, a biochemist in our department at Baylor, is using an extremely sensitive polarograph and finds definite chemical differences in certain diseases that are almost pathognomonic. We hope to present this material next year when we have more cases and are certain of our findings.

Dr. Flesch has been trying to emphasize for years that psoriatic, seborrheic, and mycosis fungoides scales are different chemically, and various diseases may be diagnosed chemically. In medicine there are many chemical tests that have been of great value to clinicians. It may be that dermatology is embarking on a new era in which chemistry will be an aid to other means of diagnosing skin problems.

Dr. Richard K. Winkelmann (Rochester, Minn.): I should like to know if Dr. Flesch still believes that there is association between parakeratosis and gram-staining. I got the impression that he meant to imply the staining is due to polysaccharides and not due to parakeratosis. I have recently described a night blue affinity for keratin. This produces much the same picture as the ninhydrin-Schiff reaction and gram-stain reaction that Dr. Flesch just presented.

Dr. Peter Flesch (in closing): I would like to thank the discussers for their comments.

Dr. Lever's remark is very apt. It is very likely that the substance in the cell walls of the epidermis, as described by Braun-Falco, is identical with the material we have studied. In case I have not made myself entirely clear, our present conception is that in normal as well as in abnormal horny layer formation the epidermal cells synthesize mucopolysaccharides. However, under normal conditions, this material decom-

poses to such an extent that in the horny layer even most of the building stones are lost. On the other hand, in pathologic keratinization, the Gram-positive material is decomposed to a much lesser extent and therefore it is possible to recover the building stones both in combined and free state. Extensive hydrolysis is needed before these substances may be detected. The idea that the pluripotentiality of the epidermal cells includes the manufacturing of mucinous materials of different composition fits in well with recent findings of Hirsch and Helwig who observed the production of cartilage by epidermal cells in their cases of syringochondroma and also with recent electron microscopic studies of epidermis which strongly favor the view that keratohyalin is deposited around the tonofilaments prior to the full hardening of the fibrils.

I am very pleased to hear about Dr. Knox's findings and am looking forward to learning more about them.

In answer to Dr. Winkelmann, it depends on how one defines parakeratosis. Persistence of Gram-staining throughout the horny layer was observed in all cases of parakeratosis, and also in non-parakeratotic horny layers. The positive staining of the cells around the sweat ducts in the horny layer of the soles is a good example of persistence of Gram-staining in non-parakeratotic horny cells.